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APPLICATION NUMBER: *60/523,119*
FILING DATE: *November 19, 2003*

PCT/DK04/00799

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FEE RECORD SHEET

11/21/2003 MBIZUNES 00000033 60523119

01 FC:1005 160.00 OP

PTO-1556
(5/87)

*U.S. Government Printing Office: 2002 — 489-267/69033

13281 U.S.PTO

Attorney Docket Number:	60820.000005

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31281 U.S.PTO 60/523119

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Additional inventors are being named on page 2 attached hereto.

TITLE OF THE INVENTION (280 characters max)

PROTEINS BELONGING TO THE BCL-2 FAMILY AND FRAGMENTS THEREOF, AND THEIR USE IN CANCER PATIENTS

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ENCLOSED APPLICATION PARTS (check all that apply)

☒ Specification — Number of Pages **27**

☐ Small Entity Status Claimed As:
- ☐ Independent Inventor
- ☐ Small Business Concern
- ☐ Nonprofit Organization
- ☐ Non-Inventor Supporting Claim By Another

☒ Drawing(s) — Number of Sheets **2**

☐ Other (specify) ______

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Respectfully submitted,

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60820.000002 WASHINGTON 386256v1

PROTEINS BELONGING TO THE Bcl-2 FAMILY AND FRAGMENTS THEREOF, AND THEIR USE IN CANCER PATIENTS

FIELD OF THE INVENTION

The present invention relates generally to the field of cancer prophylaxis and therapy. In particular there are provided isolated apoptosis regulating proteins or peptide fragments thereof that are capable of eliciting anti-cancer immune responses. Specifically, the use of such proteins belonging to the Bcl-2 protein family and immunogenic peptide fragments hereof in cancer treatment, diagnosis and prognosis is provided.

TECHNICAL BACKGROUND AND PRIOR ART

The development of resistance by cancer cells to a wide variety of chemotherapeutic agents poses a major obstacle in the successful treatment of cancer. Drug resistance is observed in a broad range of cancer cell types. Many mechanisms contribute to drug resistance, including drug inactivation, extrusion of the drug by cell membrane pumps, mutations of drug targets, and failure to initiate apoptosis. Prevention of apoptosis can result from a variety of conditions, including retention of the mitochondrial membrane potential and cytokine stimulation.

The search for proteins responsible for drug-resistant phenotypes has implicated the antiapoptotic molecule Bcl-2. Overexpression of Bcl-2 plays a role in the development of drug resistance in leukaemia and other apoptosis-prone tumours and, consequently, a poor prognosis in various human cancers. Bcl-2 belongs to a family of proteins, the Bcl-2 family, the members of which regulate apoptosis. The family includes both proapoptotic and antiapoptotic members. Although a precise understanding of how Bcl-2 exerts its antiapoptotic effects remains elusive, it has been found to be overexpressed in many cancers including lung, colorectal, prostate, and breast cancers as well as in leukaemia's and lymphomas.

Thus, Bcl-2 is a critical cellular factor, as increased expression levels of that protein confers resistance to apoptotic stimuli, thereby contributing to the pathogenesis and progression of cancer.

The identification of tumour antigens whose expression is essential for the survival of tumour cells is a new avenue to prevent antigen loss variants emerging due to selection, particularly during therapy. Thus, these characteristics suggest Bcl-2 as a suitable target for immunotherapy against a range of cancer diseases. In the studies leading to the present invention, the inventors searched for and detected spontaneous T-cell reactivity in PBL against Bcl-2 derived peptides in breast cancer patients using an ELISPOT assay.

The process by which the mammalian immune system recognises and reacts to foreign or alien materials is a complex one. An important facet of the system is the T-cell response. This response requires that T cells recognise and interact with complexes of cell surface molecules referred to as human leukocyte antigens (HLA) constituting the human major histocompatibility complex (MHC), and peptides. The peptides are derived from larger molecules, which are processed by the cells, which in turn present the HLA/MHC molecule. The interaction of T cells and complexes of HLA/peptide is restricted, requiring a T cell that is specific for a particular combination of an HLA molecule and a peptide. If a specific T cell is not present, there is no T-cell response even if its partner complex is present. Similarly, there is no response if the specific complex is absent, but the T cell is present.

The mechanism by which T cells recognise cellular abnormalities has also been implicated in cancer. E.g. in WO92/20356, a family of genes is disclosed which are processed into peptides which, in turn, are expressed on cells surfaces, and can lead to lysis of the tumour cells by specific CTLs. These genes are referred to as the MAGE family and are said to code for "tumour rejection antigen precursors" or "TRAP" molecules, and the peptides derived therefrom are referred to as "tumour rejection antigens" or "TRAs".

In WO 94/05304, nonapeptides are disclosed which bind to the HLA-A1 molecule. Thisr reference discloses that, given the known specificity of particular peptides for particular HLA molecules, one should expect a particular peptide to bind one HLA molecule, but not others. This is significant, as different individuals possess different HLA phenotypes. As a result, while identification of a particular peptide as being a partner for a specific HLA molecule has diagnostic and therapeutic ramifications, these are only relevant for individuals with that particular HLA phenotype.

Thus, it is well established that peptide epitopes derived from tumour associated antigens (TAAs) can be recognised as antigens by cytotoxic T lymphocytes (CTLs) in the context of MHC molecules. However, although it is generally accepted that most if not all, tumours are antigenic, only a few are indeed immunogenic in the sense that tumour progression is readily controlled by the immune system.

To overcome this limitation, several immunotherapeutic studies have been initiated, e.g. vaccinations with TAA-derived peptides. For melanoma, the tumour for which the largest number of CTL-defined TAAs has been characterised, powerful CTL responses against antigens have been induced by vaccination and some patients experienced a complete remission of their disease. However, most of the peptide epitopes used in these vaccination trials are melanocyte specific, and these peptides cannot be applied for tumours of non-melanocyte origin. Furthermore, expression of these TAAs is heterogeneous among tumours from different patients and can even vary among metastases obtained from one patient. However, during the last couple of years a number of tumour specific peptide antigens, which are expressed in a number of different cancers, have been identified, i.e. HER-2, Muc-1 and telomerase.

Apoptosis is a genetic program of cellular suicide, and inhibition of apoptosis has been suggested to be an important mechanism involved in cancer formation by extending the life span of cells favouring the accumulation of transforming mutations. Survivin is a recently identified member of the family of inhibitors of apoptosis proteins (IAPs). In a global gene expression analysis of about 4 million transcripts, survivin was identified as one of the top genes invariably up-regulated in many types of cancer but not in normal tissue (48). Solid malignancies overexpressing survivin include lung, colon, breast, pancreas, and prostate cancer as well as haematopoietic malignancies. Additionally, series of melanoma and non-melanoma skin cancers have been reported to be invariably survivin positive. The overexpression of survivin in most human cancers suggests a general role of apoptosis inhibition in tumour progression, a notion substantiated by the observation that in the case of colorectal and bladder cancer, as well as neuroblastoma, expression of survivin was associated with an unfavourable prognosis. In contrast, survivin is undetectable in normal adult tissues. These characteristics qualify survivin as a suitable TAA for both diagnostic and therapeutic purposes.

Thus, during the last decade a large number of TAAs have been identified which are recognised by CTLs in a major histocompatibility complex (MHC)-restricted fashion. As survivin is overexpressed in most human cancers and inhibition of its function results in increased apoptosis, this protein may serve as a target for therapeutic CTL responses. The survivin protein and the potential diagnostic and therapeutic use hereof are disclosed in (1) and US 6.245.523, which are incorporated herein by reference. Survivin is a 16.5 kDa cytoplasmic protein containing a single BIR and a highly charged carboxy-terminal coiled coil region instead of a RING finger, which inhibits apoptosis induced by growth factor (IL-3) withdrawal when transferred in B cell precursors. The gene coding for survivin is nearly identical to the sequence of Effector Cell Protease Receptor-1 (EPR-1), but oriented in the opposite direction, thus suggesting the existence of two separate genes duplicated in a head-to-head configuration. Accordingly, survivin can be described as an antisense EPR-1 product. Functionally, inhibition of survivin expression by up-regulating its natural antisense EPR-1 transcript results in massive apoptosis and decreased cell growth.

US 6.245.523 discloses the isolation of purified survivin and it provides nucleic acid molecules that encode the survivin protein, and antibodies and other molecules that bind to survivin. US 6.245.523 also discloses anti-apoptotically active fragments of the survivin protein and variants hereof wherein an amino acid residue has been inserted N- or C-terminal to, or within, the disclosed survivin sequence. It is specifically disclosed that such peptides should contain key functional residues required for apoptosis, i.e. Trp at position 67, Pro at position 73 and Cys at position 84.

During the past decade ~~numerous clinical trials~~ have shown the feasibility of peptide specific vaccination to induce anti-tumor T-cell responses in cancer patients. The clinical course of the patients, however, was in most cases not improved. This discrepancy has in numerous cases been explained by immune escape mechanisms of the tumour cells. For therapeutic strategies targeting antigens that play an insignificant role in cancer growth,

the selection of antigen deficient cancer cells is a well-recognised limitation. Thus, the attractiveness of using a Bcl-2 protein family member as a target for vaccination is based on the fact that immune escape by down regulation or loss of expression of this protein would impair sustained tumour growth. Since elevated expression of Bcl-2 protein family members in cells is correlated with drug resistance, the combination of a Bcl-2-based immunotherapy with cytotoxic chemotherapy might be an effective approach to treat cancer.

In the case of breast cancer patients, however, a paradoxical role of Bcl-2 protein has been observed. In primary breast tumours Bcl-2 negativity has been associated with a worse clinical outcome. Additionally, it has been reported that overexpression of Bcl-2 protein is correlated with oestrogen receptor-positive tumours mediated by oestrogen receptor response elements in the promoter region of the Bcl-2 gene. The prognosis of oestrogen-positive tumours is more favourable than that of oestrogen receptor-negative tumours. Several possible explanations for these seemingly paradoxical results have been suggested, e.g. inhibitory effects of Bcl-2 on cell proliferation, regulation of Bcl-2 expression by oestrogen, and/or the presence of Bcl-2 antagonists that inhibit its cytoprotective function. However, an additional explanation might be that the immune system reacts strongly against Bcl-2 positive cells as described in this study.

Still, the above studies also showed that overexpression of Bcl-2 in breast cancer is correlated with drug resistance, and that downregulation of Bcl-2 by antisense oligonucleotides modulates drug sensitivity in association with apoptosis. Furthermore, gene transfection of Bcl-2 into breast cancer cell lines has uniformly resulted in enhanced resistance to apoptosis. In addition, it has been described that the presence of another inhibitor of apoptosis, the protein survivin in breast carcinoma was strongly associated with expression of Bcl-2 and with reduced apoptotic index (AI) and poor overall survival. A similar association between survivin and Bcl-2 has been described in neuroblastoma, gastric cancer, colorectal cancer, and high-grade non-Hodgkin's lymphoma. Thus, in breast carcinoma as in most other human cancers, inhibition of apoptosis is a general feature, and expression of anti-apoptosis genes, e.g. survivin and/or Bcl-2 genes, may cause more pronounced antiapoptotic effects, as reflected in reduced apoptotic index. Recently, is has been shown that survivin is the target for spontaneous T-cell reactivity in patients with various cancers. These initial findings have later been confirmed and strengthened (by ourselves and others). Thus, a seemingly promising immunotherapeutic strategy would be to target both Bcl-2 and survivin in the cohort of cancer patients where an association between these proteins and a poor prognosis has been reported.

The present invention is based on the discovery that MHC Class I restricted peptides can be derived from a different class of apoptosis regulating proteins, i.e. the Bcl-2 protein familiy, which are capable of binding to MHC Class I HLA molecules and thereby eliciting CTL immune responses in patients suffering cancer diseases. These findings strongly suggest that proteins belonging to the Bcl-2 protein family acts as TRAP molecules, which are processed *in vivo* by cells into peptides having TRA functionality. Evidently, these

findings open the way for novel therapeutic and diagnostic approaches which may be generally applicable in the control of cancer diseases.

SUMMARY OF THE INVENTION

Accordingly, the present invention pertains is a first aspect to an isolated protein belonging to the Bcl-2 protein family or an immunogenically active peptide fragment hereof for use as a medicament in the prevention or treatment of a cancer.

In a further aspect, the invention provides a pharmaceutical composition comprising the above protein and/or peptide fragment of the invention.

In still further aspects the invention relates to a diagnostic kit for *ex vivo* or *in situ* diagnosis of the presence in a cancer patient of T cells in PBLs or in tumor tissue that are reactive with a Bcl-2 protein family member, the kit comprising the peptide fragment of the invention as defined above; a complex of a peptide fragment of the invention and a Class I HLA molecule or a fragment of such molecule.

It is also an objective of the invention to provide a method of detecting in a cancer patient the presence of a Bcl-2 protein family member reactive T cells, the method comprising contacting a tumour tissue or a blood sample with a complex of the invention as defined above an detecting binding of the complex to the tissue or the blood cells.

Additionally, there is provided a molecule that is capable of binding specifically to a peptide fragment of the invention and a molecule that is capable of blocking such binding.

In another aspect the invention pertains to a method of treating a cancer disease, the method comprising administering to a patient suffering from the disease an effective amount of the pharmaceutical composition of the invention, the molecule of the invention that is capable of binding specifically to a peptide fragment of the invention and/or a molecule of the invention that is capable of blocking such binding.

In yet another aspect the invention provides the use of the protein or peptide fragment as defined herein in the manufacturing of a medicament for the treatment of a cancer disease.

DETAILED DISCLOSURE OF THE INVENTION

It is a major objective of the present invention to provide isolated proteins belonging to the Bcl-2 protein family or an immunologically active peptide fragment hereof for use as a medicament in the prevention or treatment of a cancer.

The Bcl-2 protein family includes several proteins, which regulate apoptosis. This family includes both proapoptoctic and antiapoptotic members. In the present specification the potential of this protein family as pharmaceutically or diagnostically active substances in cancer has been studied with particular reference to the Bcl-2 protein. However, it seems very likely that immune responses similar to those observed against the Bcl-2 protein or fragments hereof exist or can be introduced in cancer patients against other members of the Bcl-2 protein family, e.g. other antiapoptotic proteins like Mcl-1 or Bcl-X_L, which are also related to drug resistance and over-expression in cancer. Accordingly, the invention pertains to any member of the Bcl-2 protein family that is capable of eliciting immune responses in cancer patients including Bcl-2, Mcl-1, Bcl-w and Bcl-X_L.

Since a number of human cancers express high levels of Bcl-2 and other members of the Bcl-2 family, immunotherapeutic strategies aiming at these antigens may have broad clinical applications. The major concern of such an approach would be the induction of auto-reactive immune responses. Thus, the future of vaccination based on members of this protein family will depend on both the therapeutic efficacy and on the type of side effects that may follow immunisation. When peptides derived from melanocyte differentiation antigens were first used to treat patients with stage IV melanoma it was envisioned that this might lead to pronounced destruction of melanocytes, which in turn would manifest itself clinically, e.g. as, vitiligo or retinitis. However, clinical experience demonstrated that the incidence of vitiligo in patients receiving vaccinations was not significantly higher than the incidence of melanoma associated hypopigmentation in patients receiving other forms of therapy. Additionally, no serious side-effects have been reported in various vaccination trials against self-antigens.

In one useful embodiment, there are provided novel MHC Class I-restricted peptide fragments (also referred to herein as "peptides") which are characterised by having at least one of several features, one of which is the ability to bind to the Class I HLA molecule to which it is restricted at an affinity as measured by the amount of the peptide that is capable of half maximal recovery of the Class I HLA molecule (C_{50} value) which is at the most 50 μM as determined by the assembly binding assay as described herein. This assembly assay is carried out as described previously (2), and it is based on stabilisation of the HLA molecule after loading of peptide to the peptide transporter deficient cell line T2. Subsequently, correctly folded stable HLA heavy chains are immunoprecipitated using conformation dependent antibodies and the peptide binding is quantitated.

This assay provides a simple means of screening candidate peptides for their ability to bind to a given HLA allele molecule at the above affinity. In preferred embodiments, the peptide fragment of the invention in one having a C_{50} value, which is at the most 30 μM, such as a C_{50} value, which is at the most 20 μM including C_{50} values of at the most 10 μM, at the most 5 μM and at the most 2 μM.

As mentioned above, the HLA system represents the human major histocompatibility (MHC) system. Generally, MHC systems control a range of characteristics: transplantation

antigens, thymus dependent immune responses, certain complement factors and predisposition for certain diseases. More specifically, the MHC codes for three different types of molecules, i.e. Class I, II and III molecules, which determine the more general characteristics of the MHC. Of these molecules, the Class I molecules are so-called HLA-A, HLA-B and HLA-C molecules that are presented on the surface of most nucleated cells and thrombocytes.

The peptides of the present invention are characterised by their ability to bind to (being restricted by) a particular MHC Class I HLA molecule. Thus, in one embodiment the peptide is one which is restricted by a MHC Class I HLA-A molecule including HLA-A1, HLA-A2, HLA-A3, HLA-A9, HLA-A10, HLA-A11, HLA-Aw19, HLA-A23(9), HLA-A24(9), HLA-A25(10), HLA-A26(10),, HLA-A28, HLA-A29(w19), HLA-A30(w19), HLA-A31(w19), HLA-A32(w19), HLA-Aw33(w19), HLA-Aw34(10), HLA-Aw36, HLA-Aw43, HLA-Aw66(10), HLA-Aw68(28), HLA-A69(28). More simple designations are also used throughout the literature, where only the primary numeric designation is used, e.g. HLA-A19 or HLA-A24 instead of HLA-Aw19 and HLA-A24(49), respectively. In specific embodiments, the peptide of the invention is restricted a MHC Class I HLA species selected from the group consisting of HLA-A1, HLA-A2, HLA-A3, HLA-A11 and HLA-A24.

The peptides of the invention may e.g. be derived from known sequences of a Bcl-2 protein family member (3). The selection of peptides potentially having the ability to bind to a particular HLA molecule can be made by the alignment of known sequences that bind to a given particular HLA molecule to thereby reveal the predominance of a few related amino acids at particular positions in the peptides. Such predominant amino acid residues are also referred to herein as "anchor residues" or "anchor residue motifs". By following such a relatively simple procedure based on known sequence data that can be found in accessible databases, peptides can be derived from the Bcl-2 protein family molecule which are likely to bind to the particular HLA molecule. Representative examples of such analyses for a range of HLA molecules are given in the below table:

HLA allele	Position 1	Position 2	Position 3	Position 5	Position 6	Position 7	C-terminal
HLA-A1		T,S	D,E			L	Y
HLA-A2		L, M			V		L,V
HLA-A3		L,V,M	F,Y				K, Y, F
HLA-A11		V,I,F,Y	M,L,F,Y,I				K, R
HLA-A23		I,Y					W,I
HLA-A24		Y		I,V	F		I,L,F
HLA-A25		M,A,T	I				W
HLA-A26	E,D	V,T,I,L,F			I,L,V		Y,F
HLA-A28	E,D	V,A,L					A,R
HLA-A29		E					Y,L
HLA-A30		Y,L,F,V					Y

HLA-A31			L,M,F,Y				R
HLA-A32		I,L					W
HLA-A33		Y,I,L,V					R
HLA-A34		V,L					R
HLA-A66	E,D	T,V					R,K
HLA-A68	E,D	T,V					R,K
HLA-A69		V,T,A					V,L
HLA-A74		T					V,L
HLA-B5		A,P	F,Y				I,L
HLA-B7		P					L,F
HLA-B8			K	K,R			L
HLA-B14		R,K					L,V
HLA-B15 (B62)		Q,L,K,P, H,V,I,M, S,T					F,Y,W
HLA-B17							L,V
HLA-B27		R					Y, K,F,L
HLA-B35		P					I, L, M, Y
HLA-B37		D,E					I,L,M
HLA-B38		H	D,E				F,L
HLA-B39		R,H					L,F
HLA-B40 (B60,61)		E	F,I,V				L,V,A,W, M,T,R
HLA-B42		L,P					Y,L
HLA-B44		E					F,Y,W
HLA-B46		M,I,L,V					Y,F
HLA-B48		Q,K					L
HLA-B51		A,P,G					F,Y,I,V
HLA-B52		Q	F,Y				I,V
HLA-B53		P					W,F,L
HLA-B54		P					
HLA-B55		P					A,V
HLA-B56		P					A,V
HLA-B57		A,T,S					F,W,Y
HLA-B58		A,T,S					F,W,Y
HLA-B67		P					L
HLA-B73		R					P
HLA-Cw1		A,L					L
HLA-Cw2		A,L					F,Y
HLA-Cw3		A,L					L,M
HLA-		Y,P,F					L,M,F,Y

Cw4							
HLA-Cw6							L,I,V,Y
HLA-Cw6		Y					L,Y,F
HLA-Cw8		Y					L,I,
HLA-Cw16		A,L					L,V

Thus, as an example, nonapeptides potentially having the ability to bind to HLA-A1 would have one of the following sequences: Xaa-T-D-Xaa-Xaa-Xaa-L-Xaa-Y, Xaa-T-E-Xaa-Xaa-Xaa-L-Xaa-Y; Xaa-S-D-Xaa-Xaa-Xaa-L-Xaa-Y or Xaa-S-E-Xaa-Xaa-Xaa-L-Xaa-Y (Xaa indicating any amino acid residue). In a similar manner, sequences potentially having the ability to bind to any other HLA molecule can be designed.

It will be appreciated that the person of ordinary skill in the art will be able to identify further "anchor residue motifs" for a given HLA molecule.

Thus, in useful embodiments, the peptides of the invention include peptides, the sequences of which comprise, for each of the specific HLA alleles listed in the table, any of the amino acid residues as indicated in the table.

Thus, a simple approach to identifying peptides of the invention includes the following steps: selecting a particular HLA molecule, e.g. one occurring at a high rate in a given population, carrying out an alignment analysis as described above to identify "anchor residue motifs" in the Bcl-2 protein family protein, isolating or constructing peptides of a suitable size that comprise one or more of the identified anchor residues and testing the resulting peptides for (i) capability to bind to the particular HLA molecule using the assembly assay as described herein, (ii) the capability of the peptides to elicit INF-γ-producing cells in a PBL population of a cancer patient at a frequency of at least 1 per 10^4 PBLs as determined by an ELISPOT assay as described herein, and/or (iii) the capability of the peptides to detect *in situ* in a tumour tissue CTLs that are reactive with the epitope peptides being tested.

In specific embodiments, the peptide of the invention is an HLA-A2 restricted Bcl-2-derived peptide having a sequence selected from the following: ALVGACITL (SEQ ID NO:1), ALSPVPPVV (SEQ ID NO:2), SLALVGACI (SEQ ID NO:3), KTLLSLALV (SEQ ID NO:4), LLSLALVGA (SEQ ID NO:5), WLSLKTLLSL (SEQ ID NO:6), AAAGPALSPV (SEQ ID NO:7), PLFDFSWLSL (SEQ ID NO:8), ~~FTARGRFATV (SEQ~~ ID NO:9), YLNRHLHTWI (SEQ ID NO:10), NIALWMTEYL (SEQ ID NO:11).

In further useful embodiments, the peptide of the invention is a peptide, which is restricted by a MHC Class I HLA-B molecule including any of the following: HLA-B5, HLA-B7, HLA-B8,

HLA-B12, HLA-B13, HLA-B14, HLA-B15, HLA-B16, HLA-B17, HLA-B18, HLA-B21, HLA-Bw22, HLA-B27, HLA-B35, HLA-B37, HLA-B38, HLA-B39, HLA-B40, HLA-Bw41, HLA-Bw42, HLA-B44, HLA-B45, HLA-Bw46 and HLA-Bw47. In specific embodiments, the MHC Class I HLA-B species to which the peptide of the invention is capable of binding is selected from HLA-B7, HLA-B35, HLA-B44, HLA-B8, HLA-B15, HLA-B27 and HLA-B51.

In further useful embodiments, the peptide of the invention is a peptide, which is restricted by a MHC Class I HLA-C molecule including any of the following: HLA-Cw1, HLA-Cw2, HLA-Cw3, HLA-Cw4, HLA-Cw5, HLA-Cw6, HLA-Cw7 and HLA-Cw1.

Preferably, the peptide fragment of the invention comprises less than 50 amino acid residues, and more preferably it comprises at the most 20 amino acid residues, such as at the most 10 amino acid residues. In specific embodiments, the peptide is a heptapeptide, an octopeptide, a nonapeptide, a decapeptide or an undecapeptide.

The peptide of the invention is, as mentioned above, derived from a Bcl-2 protein family member or a fragment hereof. The protein from which the peptide can be derived can be any Bcl-2 protein family member from any animal species in which the protein is expressed. In preferred embodiments, the starting protein is from a mammal species including a rodent species, rabbit and a primate species such as humans. Based on the sequence of the selected protein, the peptide of the invention is derived by any appropriate chemical or enzymatic treatment of the protein starting material that results in a peptide of a suitable size as indicated above, or it can be synthesised by any conventional peptide synthesis procedures with which the person of ordinary skills in the art is familiar.

The peptide of the invention may have a sequence which is a native sequence of the Bcl-2 protein family member from which is derived. However, peptides having a higher affinity to any given HLA molecule may be derived from such a native sequence by modifying the sequence by substituting, deleting or adding at least one amino acid residue, e.g. on the basis of the procedure described above whereby anchor residue motifs in respect of the given HLA molecule are identified.

A significant feature of the peptide of the invention is its capability to recognise or elicit INF-γ -producing responder T cells, i.e. cytotoxic T cells (CTLs) that specifically recognise the particular peptide in a PBL population or tumour cells of a cancer patient (target cells). This activity is readily determined by subjecting PBLs or tumour cells from a patient to an ELISPOT assay as described in reference (4) and in the following example. Prior to the assay, it may be advantageous to stimulate the PBL population or the tumour cells to be assayed by contacting the cells with the peptide to be tested. Preferably, the peptide is capable of eliciting or recognising INF-γ -producing T cells at a frequency of at least 1 per 10^4 PBLs as determined by an ELISPOT assay as used herein. More preferably the frequency is at least 5 per 10^4 PBLs, most preferably at least 10 per 10^4 PBLs, such as at least 50 or 100 per 10^4 PBLs.

The ELISPOT assay represents a strong tool to monitor Bcl-2 family-derived peptide specific T-cell responses. However, although it has been shown that ELISPOT reactivity in most cases correlates with the capacity of the CTLs to lyse target cells, the conclusive evidence for this notion can only be given directly. Therefore, a major implication of the findings herein is that the peptides of the invention may be expressed and complexed with HLA molecules on cancer cells. This renders these cancer cells susceptible to destruction by CTLs and emphasizes the potential usefulness of Bcl-2 family protein immunization to control the growth of neoplasms. The presence of spontaneous CTL-responses in PBLs from breast cancer patients to HLA-restricted Bcl-2-derived peptide epitopes substantiates the immunotherapeutic potential of these tumour antigens not only in breast cancer patients, but also, as Bcl-2 protein family member are overexpressed in many cancers including lung, colorectal, prostate cancers and in leukaemia and lymphomas, in a broad range of cancer diseases.

Accordingly, in another preferred embodiment the peptide of the invention is capable of eliciting INF-γ -producing cells in a PBL population of a patient having a cancer disease where a Bcl-2 protein family is expressed including a haematopoietic malignancy e.g chronic lymphatic leukaemia and chronic myeloid leukaemia, melanoma, breast cancer, cervix cancer, ovary cancer, lung cancer, colon cancer, pancreas cancer and prostate cancer.

In addition to their capacity to elicit immune responses in PBL populations it is also contemplated that the peptides of the invention are capable of eliciting cytolytic immune responses *in situ*, i.e. in solid tumour tissues. This may be demonstrated by providing HLA-peptide complexes, e.g. being multimerised and being provided with a detectable label, and using such complexes for immunohistochemistry stainings to detect in a tumour tissue CTLs that are reactive with the epitope peptide of the invention. Accordingly, a further significant feature of the peptide of the invention is that it is capable of *in situ* detection in a tumour tissue of CTLs that are reactive with the epitope peptide.

It is also contemplated that the peptides of the invention, in addition to their capacity to bind to HLA molecules resulting in the presentation of complexes of HLA and peptides on cell surfaces, which complexes in turn act as epitopes or targets for cytolytic T cells, may elicit other types of immune responses, such as B-cell responses resulting in the production of antibodies against the complexes and/or a Delayed Type Hypersensitivity (DTH) reaction. The latter type of immune response is defined as a redness and palpable induration at the site of injection of the peptide of the invention.

It is evident that the findings of the present invention provide the basis for therapeutic as well as diagnostic applications of the protein or the peptide fragment of the invention.

Accordingly, in a further aspect the present invention provides a pharmaceutical composition comprising the protein or the peptide fragment of the invention, in particular a

pharmaceutical composition which, when it is administered to a cancer patient, is capable of eliciting an immune response against the cancer disease including eliciting the production in the vaccinated patient of effector T cells having a cytotoxic effect against the cancer cells.

As it is well known, that the different HLA molecules are of different prevalence in the major human populations, there is a requirement of identifying peptide epitopes restricted to several HLA class I molecules to extend the patient cohort that can be treated according to the methods of the present invention. The characterisation of multiple Bcl-2 epitopes with different HLA restriction elements broadens the clinical potential of this target antigen in two important ways: (i) It increases the number of patients eligible for immunotherapy based on Bcl-2 derived peptides. The HLA-A2 antigen is expressed by around 50 % of the Caucasian and Asian populations, the HLA-A1 and HLA-A3 antigens are both expressed by around 25 % of Caucasians and 5 % of Asians, whereas the HLA-A11 antigen is expressed by around 15 % of Caucasians and 30 % of Asians. Even though these numbers cannot be added up due to co-expression, a combination of peptides restricted by a multiplicity of these would certainly encompass most cancer patients, (ii) The collective targeting of several restriction elements in each patient is likely to decrease the risk of immune escape by HLA-allele loss. Loss of a single HLA allele is a significant component of MHC alterations described for cancer cells, whereas total loss of Class I expression is a rather infrequent event. Thus, with the identification of Bcl-2 epitopes restricted to different HLA alleles, it would be possible to target more than one HLA-molecule simultaneously in patients with allelic overlap.

Thus, it would be possible to develop highly immunogenic multi-epitope vaccines. Preferably, such vaccines should be designed so as to facilitate a simultaneous delivery of the best-suited Bcl-2-derived peptides optionally in combination with other suitable peptides and/or adjuvants as described hereinafter. The present invention encompasses such multiepitope vaccines comprising Bcl-2-derived peptides optionally in combination with further proteins or peptides fragments not belonging to or derived from the Bcl-2 protein family and/or adjuvants as described hereinafter and/or clas II-MHC restricted epitopes as described below.

There has been an increased focus on eliciting tumor-specific T helper cell immunity, i.e., vaccinating with class II-MHC restricted epitopes despite the fact that tumors generally do not express class II MHC. This is based on the recent finding that the induction and efficacy of the vaccine-induced anti-tumor response in many cases requires the cooperation of tumor-specific CD4 positive T_h cells. Thus, an important factor driving the development of vaccines having a more complex composition is the desire to target multiple tumor antigens e.g. by designing vaccines comprising or encoding a collection of carefully selected CTL and T_h cell epitopes.

Obviously, multi-epitope vaccines constitute an efficient way to raise immunity against epitopes derived from several different antigens without the need for introducing (genes

encoding) potentially hazardous proteins such as oncoproteins. Such vaccines also permit selective induction of immunity against subdominant and cryptic T cell epitopes, which can be especially important in the case of tumor-associated autoantigens for which tolerance may exist for the epitopes that are prominently presented in normal tissues. Furthermore, antigen-presenting cells may fail to present certain epitopes that are expressed on tumor cells because of functional differences between the immunoproteasomes of antigen-presenting cells and the 'constitutive' proteasomes present in most tumor cells. In the case of peptide-based vaccines, such epitopes can be administered in an 'MHC-ready' form, which enables presentation through exogenous loading independently of antigen uptake and processing by host antigen-presenting cells.

As the peptides of the invention are relatively small molecules it may be required in such compositions to combine the peptides with various materials such as adjuvants, to produce vaccines, immunogenic compositions, etc. Adjuvants, broadly defined, are substances which promote immune responses. Frequently, the adjuvant of choice is Freund's complete or incomplete adjuvant, or killed *B. pertussis* organisms, used e.g. in combination with alum precipitated antigen. A general discussion of adjuvants is provided in Goding, Monoclonal Antibodies: Principles & Practice (2nd edition, 1986) at pages 61-63. Goding notes, however, that when the antigen of interest is of low molecular weight, or is poorly immunogenic, coupling to an immunogenic carrier is recommended. Examples of such carrier molecules include keyhole limpet haemocyanin, bovine serum albumin, ovalbumin and fowl immunoglobulin. Various saponin extracts have also been suggested to be useful as adjuvants in immunogenic compositions. Recently, it has been proposed to use granulocyte-macrophage colony stimulating factor (GM-CSF), a well known cytokine, as an adjuvant (WO 97/28816).

Accordingly, the invention encompasses a therapeutic composition further comprising an adjuvant substance including any of the above or combinations thereof. It is also contemplated that the antigen, i.e. the peptide of the invention and the adjuvant can be administered simultaneously or separately in any appropriate sequence.

The choice of antigen in the pharmaceutical composition of the invention will depend on parameters determinable by the person of skill in the art. As it has been mentioned, each of the different peptides of the invention is presented on the cell surfaces by a particular HLA molecule. As such, if a subject to be treated is typed with respect to HLA phenotype, a peptide/peptides are selected that is/are known to bind to that particular HLA molecule.

Alternatively, the antigen of interest is selected based on the prevalence of the various HLA phenotypes in a given population. As an example, HLA-A2 is the most prevalent phenotype in the Caucasian population, and therefore, a composition containing a survivin derived peptide binding to HLA-A2 will be active in a large proportion of that population. However, the composition of the invention may also contain a combination of two or more survivin derived peptides, each interacting specifically with a different HLA molecule so as to cover a larger proportion of the target population. Thus, as examples, the pharmaceuti-

cal composition may contain a combination of a peptide restricted by a HLA-A molecule and a peptide restricted by a HLA-B molecule, e.g. including those HLA-A and HLA-B molecules that correspond to the prevalence of HLA phenotypes in the target population, such as e.g. HLA-A2 and HLA-B35. Additionally, the composition may comprise a peptide restricted by an HLA-C molecule.

It is contemplated that useful immunogenic compositions of the invention, in addition to a Bcl-2 protein family member derived peptide as defined herein may comprise an immunologically effective amount of the Bcl-2 protein family member as such as it is defined herein or an immunogenic fragment hereof.

The amount of the immunogenic peptide of the invention in the pharmaceutical composition may vary, depending on the particular application. However, a single dose of the immunogen is preferably anywhere from about 10 µg to about 5000 µg, more preferably from about 50 µg to about 2500 µg such as about 100 µg to about 1000 µg. Modes of administration include intradermal, subcutaneous and intravenous administration, implantation in the form of a time release formulation, etc. Any and all forms of administration known to the art are encompassed herein. Also any and all conventional dosage forms that are known in the art to be appropriate for formulating injectable immunogenic peptide composition are encompassed, such as lyophilised forms and solutions, suspensions or emulsion forms containing, if required, conventional pharmaceutically acceptable carriers, diluents, preservatives, adjuvants, buffer components, etc.

In a further embodiment of the invention, the pharmaceutical composition of the invention is useful for treating a cancer patient, where, during cancer progression in that patient, the cancer cells have developed a reduced susceptibility to a chemotherapeutically active anticancer drug and/or radiotherapy.

The pharmaceutical composition of the invention may advantageously comprise at least one further immunogenic protein or peptide fragment hereof selected from a protein or peptide fragment not belonging to or derived from the Bcl-2 protein family, including a protein involved in regulation of cell apoptosis or a peptide fragment derived therefrom. As one example, such a further protein or peptide is survivin as defined above, or a peptide fragment hereof. In specific embodiments, a further immunogenic survivin-derived peptide is an HLA-A2 restricted peptide having a sequence selected from the following: FLKLDRERA ($survivin_{101-109}$) (SEQ ID NO:12), TLPPAWQPFL ($survivin_{5-14}$) (SEQ ID NO:13), ELTLGEFLKL ($survivin_{95-104}$) (SEQ ID NO:14), LLLGEFLKL (SEQ ID NO:15) and LMLGEFLKL (SEQ ID NO:16). (The designations in brackets indicate the positions of the residues in the survivin protein as disclosed in US 6.245.523). LLLGEFLKL (SEQ ID NO:15) is a sequence derived from $survivin_{96-104}$ by substituting "T" in position 2 of the peptide with an "L" and LMLGEFLKL (SEQ ID NO:16) is derived from $survivin_{96-104}$ by substituting "T" in position 2 with "M". In further specific embodiments, the further immunogenic survivin-derived peptide is an HLA-B35-restricted survivin-derived peptide having a sequence selected from the following: CPTENEPDL ($survivin_{46-54}$) (SEQ ID NO:17), EPDLAQCFF ($survivin_{51-59}$) (SEQ

ID NO:18), CPTENEPDY (SEQ ID NO:19) and EPDLAQCFY (SEQ ID NO:20). (The designations in brackets indicate the positions of the residues in the survivin protein as disclosed in US 6.245.523). CPTENEPDY (SEQ ID NO:19) is a sequence derived from survivin46-54 by substituting "L" in the C-terminal of the peptide with a "Y" and EPDLAQCFY (SEQ ID NO:20) is derived from survivin$_{51-59}$ by substituting an "F" residue in the C-terminal 2 with a "Y".

In yet further embodiments, the further peptide is a HLA-A1 restricted peptide having a sequence selected from the following: Survivin$_{38-46}$ (Sur38Y9) (a C changed to a Y at P9, MAEAGFIHY)(SEQ ID NO:21), Survivin$_{47-56}$ (Sur47Y10) (a Q changed to a Y at P10, PTENEPDLAY(SEQ ID NO:22)), Survivin$_{92-101}$ (Sur92-101) (QFEELTLGEF) (SEQ ID NO:23), and Survivin$_{93-101}$ (Sur93T2 (a E changed to a T at P2, FTELTLGEF (SEQ ID NO:24)). The peptide of the invention may also be a HLA-A3 restricted peptide such as Survivin$_{18-24}$ (Sur18K10) (a F changed to a K at P10, RISTFKNWPK (SEQ ID NO:25) and/or a HLA-A11 restricted peptide such as Survivin$_{53-62}$ (Sur53-62)(DLAQCFFCFK)(SEQ ID NO:26) and/or a HLA-A2 restricted peptide such as Survivin$_{18-28}$ (Sur18-28) (RISTFKNWPFL)(SEQ ID NO:27).

Other useful further peptides includes the known apoptosis inhibitor polypeptide ML-IAP which has a rather selective expression, and is detected in melanomas. Thus, fragments of ML-IAP capable of eliciting a specific T-cell response i.e. a cytotoxic T-cell response or a helper T-cell response may optionally be included in the composition of the present invention. Useful peptide fragments of ML-IAP include ML-IAP$_{245}$ (RLQEERTCKV)(SEQ ID NO:28), ML-IAP$_{280}$ (QLCPICRAPV)(SEQ ID NO:29), ML-IAP$_{90}$ (RLASFYDWPL)(SEQ ID NO:30), ML-IAP$_{154}$ (LLRSKGRDFV)(SEQ ID NO:31), ML-IAP$_{230}$ (VLEPPGARDV)(SEQ ID NO:32), ML-IAP$_{98}$ (PLTAEVPPEL)(SEQ ID NO:33), ML-IAP$_{34}$ (SLGSPVLGL)(SEQ ID NO:34), ML-IAP$_{54}$ (QILGQLRPL)(SEQ ID NO:35), ML-IAP$_{99}$ (LTAEVPPEL)(SEQ ID NO:36), ML-IAP$_{83}$ (GMGSEELRL)(SEQ ID NO:37) and ML-IAP$_{200}$ (ELPTPRREV)(SEQ ID NO:38).

Additionally, the composition according to the present invention may be provided as a multiepitope vaccine comprising class I restricted epitope and class II restricted epitopes as defined hereinbefore.

The immunoprotective effect of the composition of the invention can be determined using several approaches e.g. as described in WO 97/28816, *supra*. A successful immune response may also be determined by the occurrence of DTH reactions after immunisation and/or the detection of antibodies specifically recognising the peptide(s) of the vaccine composition.

In preferred embodiments, the pharmaceutical composition of the invention is an immunogenic composition or vaccine capable of eliciting an immune response to a cancer disease. As used herein, the expression "immunogenic composition or vaccine" refers to a composition eliciting at least one type of immune response directed against cancer cells. Thus, such an immune response may be any of the types mentioned above: A CTL response

where CTLs are generated that are capable of recognising the HLA/peptide complex presented on cell surfaces resulting in cell lysis, i.e. the vaccine elicits the production in the vaccinated subject of effector T-cells having a cytotoxic effect against the cancer cells; a B-cell response giving rise to the production of anti-cancer antibodies; and/or a DTH type of immune response.

In useful embodiments an immunogenic response directed against a cancer disease is elicited by administering the peptide of the invention either by loading MHC class I molecules on antigen presenting cells (APCs) from the patient, by isolating PBLs from the patient and incubating the cells with the peptide prior to injecting the cells back into the patient or by isolating precursor APCs from the patient and differentiating the cells into professional APCs using cytokines and antigen before injecting the cells back into the patient. Thus, in one embodiment of the present invention, a method for treating cancer patients is one wherein the peptide is administered by presenting the peptide to the patient's antigen presenting cells (APCs) *ex vivo* followed by injecting the thus treated APCs back into the patient. There are at least two alternative ways of performing this. One alternative is to isolate APCs from the cancer patient and incubate (load) the MHC class I molecules with the peptide. Loading the MHC class I molecules means incubating the APCs with the peptide so that the APCs with MHC class I molecules specific for the peptide will bind the peptide and therefore be able to present it to T cells. Subsequently, the APCs are re-injected into the patient. Another alternative way relies on the recent discoveries made in the field of dendritic cell biology. In this case, monocytes (being dendritic cell precursors) are isolated from the patient and differentiated in vitro into professional APC (or dendritic cells) by use of cytokines and antigen. Subsequently, the in vitro generated DCs are pulsed with the peptide and injected into the patient.

Due to the fact that members of the Bcl-2 protein family appear to be expressed in a range of cancer forms, it is very likely that vaccines of the invention can be provided to control any type of cancer disease where such proteins are expressed. Thus, as examples, the vaccine composition of the invention is immunologically active against a haematopoietic malignancy including chronic lymphatic leukemia and chronic myeloid leukaemia, melanoma, breast cancer, cervix cancer, ovary cancer, lung cancer, colon cancer, pancreas cancer and prostate cancer.

From the above description, the skilled person will readily realise that the proteins and/or peptides of the invention are useful as cancer diagnostic tools. Therefore, the peptides of the invention provide the basis for developing widely applicable diagnostic and prognostic procedures in respect of cancer diseases. Thus, in other useful embodiments the composition of the invention is a composition for *ex vivo* or *in situ* diagnosis of the presence in a cancer patient, e.g. based on the detection of Bcl-2 protein family member reactive T cells among PBLs or in tumour tissue.

Accordingly, there is, in still further aspects, provided a diagnostic kit for *ex vivo* or *in situ* diagnosis of the presence in a cancer patient of Bcl-2 family member reactive T cells

among PBLs or in tumour tissue comprising one or more peptides of the invention, and a method of detecting in a cancer patient the presence of such reactive T cells, the method comprising contacting a tumour tissue or a blood sample with a complex of a peptide of the invention and a Class I HLA molecule or a fragment of such molecule and detecting binding of the complex to the tissue or the blood cells.

Another useful diagnostic or prognostic approach is based on generating antibodies in a heterologous animal species, e.g. murine antibodies directed against a human Bcl-2 protein family member-derived peptide of the invention, which can then be used, e.g. to diagnose for the presence of cancer cells presenting the peptide. For such immunisation purposes, the amount of peptide may be less than that used in the course of *in vivo* therapy, such as that mentioned above. In general, a preferred dose can range from about 1 µg to about 750 µg of peptide. It is also possible to produce monoclonal antibodies based on immunisation with a peptide of the invention. Accordingly, the present invention also relates to a molecule, in particular a monoclonal or polyclonal antibody including a fragment hereof, that is capable of binding specifically to a peptide of the invention and to a molecule that is capable of blocking such a binding, e.g. an antibody raised against the monoclonal or polyclonal antibody directed against a peptide of the invention.

In one aspect, the invention provides a complex of a peptide of the invention and a Class I HLA molecule or a fragment of such molecule, which is useful as a diagnostic reagent such as it is described *supra*. Such a complex may be monomeric or multimeric.

The present invention provides the means for alleviating or curing a cancer disease. Accordingly, it is a further aspect of the invention to provide a method of treating a cancer disease associated with the expression of a Bcl-2 protein family member, including as examples: a haematopoietic malignancy including chronic lymphatic leukaemia and chronic myeloid leukaemia, melanoma, breast cancer, cervix cancer, ovary cancer, lung cancer, colon cancer, pancreas cancer and prostate cancer, which method comprises administering to a patient suffering from the disease an effective amount of the pharmaceutical composition according to the invention, a molecule that is capable of binding specifically to a peptide of the invention and/or a molecule that is capable of blocking the binding of such a molecule.

In some cases it will be appropriate to combine the treatment method of the invention with a conventional cancer treatment such as chemotherapy, radiotheraphy, treatment with immunostimulating substances, gene therapy, treatment with antibodies and treatment using dendritic cells.

The invention will now be illustrated by the following, non-limiting examples and the drawings wherein

Fig. 1 shows identification of HLA-A2 binding peptides from Bcl-2. Class I MHC heavy chain bands were quantified using a Phosphorimager. The amount of stabilised HLA-A2

heavy chain is directly related to the binding affinity of the added peptide. The binding of the HLA-A2-restricted positive control peptide HIV Pol_{476} (black square) was compared with the peptides Bcl_{172} (black triangle), Bcl_{180} (black circle), and Bcl_{200} (white circle) and

Fig. 2 illustrates T-cell response against the peptides Bcl_{172}, Bcl_{180}, Bcl_{208}, and Bcl_{214}. PBL from 15 breast cancer patients were analysed. T-lymphocytes were stimulated once with peptide before plated at 10^5 cells per well in triplicates either without or with peptide. The average number of peptide specific spots (after subtraction of spots without added peptide) was calculated for each patient using the ImmunoSpot® Series 2.0 Analyzer (CTL Analyzers, LLC, Cleveland, US).

EXAMPLES

Materials and Methods

1. *Patients*

Peripheral blood lymphocytes (PBL) were collected from breast cancer patients. PBL were isolated using Lymphoprep separation, HLA-typed (Department of Clinical Immunology, University Hospital, Copenhagen, Denmark) and frozen in FCS with 10% DMSO. None of the patients received immunotherapy prior to sampling of blood.

2. *Assembly assay for peptide binding to MHC class I molecules*

The binding affinity of the synthetic peptides (Invitrogen, Carlsbad, CA, USA) to HLA-A2 molecules, metabolically labelled with [^{35}S]-methionine, was measured in the assembly assay, as described previously. The assay is based on peptide-mediated stabilisation of empty HLA molecules released upon cell lysis, from the TAP-deficient cell line T2. Stably folded HLA-molecules were immune-precipitated using the HLA class I-specific, conformation-dependent mAb W6/32, and separated by isoelectric focusing (IEF) gel electrophoresis. MHC heavy chain bands were quantified using the ImageGauge Phosphorimager program (FUJI photo film Co., Carrollton, TX, USA). The intensity of the band is directly related to the amount of peptide-bound class I MHC complex recovered during the assay. Subsequently, the extent of stabilisation of HLA-A2 is directly related to the binding affinity of the added peptide. The recovery of HLA-A2 was measured in the presence of 50, 5, 0.5, 0.05 μM of the relevant peptide. The C_{50} value was calculated for each peptide as the peptide concentration sufficient for half maximal stabilisation.

3. *Antigen stimulation of PBL*

To extend the sensitivity of the ELISPOT assay, PBL were stimulated once *in vitro* prior to analysis (16,17). At day 0, PBL or crushed lymph nodes were thawed and plated in 2 ml/well at a concentration of 2×10^6 cells in 24-well plates (Nunc, Denmark) in X-vivo

medium (Bio Whittaker, Walkersville, Maryland), 5% heat-inactivated human serum, and 2 mM of L-glutamine in the presence of 10 μM of peptide. Two days later 20 IU/ml recombinant interleukin-2 (IL-2) (Chiron, Ratingen, Germany) was added to the cultures. The cultured cells were tested for reactivity in the ELISPOT on day 12.

4. *ELISPOT assay*

The ELISPOT assay was used to quantify peptide epitope-specific interferon-γ releasing effector cells as described previously (4). Briefly, nitrocellulose bottomed 96-well plates (MultiScreen MAIP N45, Millipore, Hedehusene, Denmark) were coated with anti-IFN-γ antibody (1-D1K, Mabtech, Nacka, Sweden). The wells were washed, blocked by X-vivo medium, and cells added in duplicates at different cell concentrations. Peptides were then added to each well and the plates were incubated overnight. The following day, media was discarded and the wells were washed prior to addition of biotinylated secondary antibody (7-B6-1-Biotin, Mabtech). The plates were incubated for 2 hours, washed and Avidin-enzyme conjugate (AP-Avidin, Calbiochem, Life Technologies) was added to each well. Plates were incubated at RT for 1 hour and the enzyme substrate NBT/BCIP (Gibco, Life Technologies) was added to each well and incubated at RT for 5-10 min. The reaction was terminated by washing with tap-water upon the emergency of dark purple spots. The spots were counted using the ImmunoSpot® Series 2.0 Analyzer (CTL Analyzers, LLC, Cleveland, US) and the peptide specific CTL frequency could be calculated from the numbers of spot-forming cells. All assays were performed in triplicates for each peptide antigen.

5. *Results*

Binding of Bcl-2 derived peptides to HLA-A2

The amino acid sequence of the Bcl-2 protein was screened for the most probable HLA-A2 nona- and decamer peptide epitopes, using the main HLA-A2 specific anchor residues (2). Thirteen Bcl-2 derived peptides were synthesised and examined for binding to HLA-A2 by comparison with the HLA-A2 high affinity positive control epitope from HIV-1 $pol_{476-484}$ (ILKEPVHGV) (SEQ ID NO:39) by the assembly assay. The assembly assay is based on stabilisation of the class I molecule after loading of different concentrations of peptide to the TAP-deficient cell line T2. Subsequently correctly folded stable MHC heavy chains are immunoprecipitated using conformation-dependent antibodies. The extent of stabilisation of class I MHC molecules is directly related to the binding affinity of the added peptide as exemplified in Fig. 1. The peptide concentration required for half maximal recovery of class I MHC molecules (C_{50} value) were 0.7 μM for the HIV-1 $pol_{476-484}$ (Table 1). Eight Bcl-2 derived peptides bound with almost similar high affinity as the positive control; Bcl_{224}, Bcl_{85}, Bcl_{222}, Bcl_{218}, Bcl_{220}, Bcl_{214}, Bcl_{124} and Bcl_{172} (C_{50} = 0.7, 1, 1, 2, 1, 3, 1, and 2 μM, respectively) (Table 1). The peptides Bcl_{80}, Bcl_{208} and Bcl_{180} bound only with intermediate or weak affinity (C_{50} = 36, 7 and 20 μM, respectively. Two of the peptides examined (Bcl_{216}, Bcl_{200}) did not bind to HLA-A2 at all. A list of the peptides included in this study are shown in Table 1:

Table 1. Peptides examined in this study

Protein[a]	Sequence	SEQ ID NO	C_{50}(μM)[b]
HIV-1 pol_{476}	ILKEPVHGV	39	0.7
Bcl_{224}	A L V G A C I T L	1	0.7
Bcl_{85}	A L S P V P P V V	2	1
bcl_{222}	S L A L V G A C I	3	1
bcl_{218}	K T L L S L A L V	4	2
bcl_{220}	L L S L A L V G A	5	1
bcl_{214}	W L S L K T L L S L	6	3
bcl_{80}	A A A G P A L S P V	7	36
bcl_{216}	S L K T L L S L A L	40	Not binding
bcl_{208}	P L F D F S W L S L	8	7
bcl_{124}	F T A R G R F A T V	9	1
bcl_{180}	Y L N R H L H T W I	10	15
bcl_{172}	N I A L W M T E Y L	11	2
bcl_{200}	E L Y G P S M R P L	41	Not binding

[a] The value range listed in subscript indicates the position of the first amino acid in the sequence

[b] The C_{50} value is the concentration of the peptide required for half maximal binding to HLA-A2

CTL responses against BCL-2 derived peptides in chemotherapy treated breast cancer patients

Using the ELISPOT IFN-γ secretion assay, we examined the presence of specific T-cell responses against the Bcl-2 derived peptides in peripheral blood T cells from breast cancer patients. This method has previously been highly effective when identifying tumour specific CTL in cancer patients.

PBL from 15 HLA-A2 positive breast cancer patients were stimulated once *in vitro* before examination in the ELISPOT. This procedure was chosen to extend the sensitivity of the ELISPOT as described (4). Since many described CTL epitopes are in fact low affinity peptides we included all thirteen Bcl-2 deduced peptides in the first line of experiments. Responses were detected against Bcl_{172}, Bcl_{180}, Bcl_{208}, and Bcl_{214} and only data from these peptides are given in Fig. 2. Spontaneous CTL responses were detected against Bcl_{172} in PBL from eight of the patients (50%), and against Bcl_{180} in four of the patients (≈25%) (Fig. 2). However, the most frequent responses were detected against Bcl_{208} and Bcl_{214}, since twelve (≈80%) of the patients hosted a detectable CTL response against Bcl_{208} and eleven of the patients (≈75%) hosted a Bcl_{214}–response (Fig. 2).

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CLAIMS

1. An isolated protein belonging to the Bcl-2 protein family or an immunogenically active peptide fragment hereof for use as a medicament in the prevention or treatment of a cancer.

2. The protein or peptide fragment of claim 1 where the protein is selected from the group consisting of Bcl-2, Bcl-w, Mcl-1 and Bcl-X_L.

3. The protein or peptide fragment of claim 2 where the protein is Bcl-2.

4. The peptide fragment of claim 1 that is capable of eliciting a cellular immune response in a cancer patient.

5. The peptide fragment of claim 4 which is a MHC Class I-restricted peptide having at least one of the following characteristics:

(i) capable of binding to the Class I HLA molecule to which it is restricted at an affinity as measured by the amount of the peptide that is capable of half maximal recovery of the Class I HLA molecule (C_{50} value) which is at the most 50 µM as determined by the assembly binding assay as described herein,

(ii) capable of eliciting INF-γ -producing cells in a PBL population of a cancer patient at a frequency of at least 1 per 10^4 PBLs as determined by an ELISPOT assay, and/or

(iii) capable of *in situ* detection in a tumor tissue of CTLs that are reactive with the epitope peptide.

6. The peptide fragment of claim 5 having a C_{50} value, which is at the most 30 µM.

7. The peptide fragment of claim 6 having a C_{50} value, which is at the most 20 µM.

8. The peptide fragment of claim 1, which is restricted by a MHC Class I HLA-A molecule.

9. The peptide fragment of claim 8, which is restricted by a MHC Class I HLA species selected from the group consisting of HLA-A1, HLA-A2, HLA-A3, HLA-A11 and HLA-A24.

10. The peptide fragment of claim 9, which is restricted by HLA-A2.

11. The peptide fragment of claim 10, which is selected from the group consisting of ALVGACITL (SEQ ID NO:1), ALSPVPPVV (SEQ ID NO:2), SLALVGACI (SEQ ID NO:3), KTLLSLALV (SEQ ID NO:4), LLSLALVGA (SEQ ID NO:5), WLSLKTLLSL (SEQ ID NO:6), AAAGPALSPV (SEQ ID NO:7), PLFDFSWLSL (SEQ ID NO:8), FTARGRFATV (SEQ ID NO:9), YLNRHLHTWI (SEQ ID NO:10), NIALWMTEYL (SEQ ID NO:11).

12. The peptide fragment of claim 1, which is restricted by a MHC Class I HLA-B molecule.

13. The peptide fragment of claim 12, which is restricted by a MHC Class I HLA-B species selected from the group consisting of HLA-B7, HLA -B35, HLA -B44, HLA-B8, HLA-B15, HLA-B27 and HLA-B51.

14. The peptide fragment of claim 1 comprising at the most 20 amino acid residues.

15. The peptide fragment of claim 14 comprising at the most 15 amino acid residues.

16. The peptide fragment of claim 15, which is a nonapeptide or a decapeptide.

17. The protein or peptide fragment of claim 1, which is a native sequence isolated or derived from a mammal species.

18. The protein or peptide fragment of claim 17 where the protein is a human protein.

19. The protein or peptide fragment of claim 1, which is derived from a native Bcl-2 protein family member sequence by substituting, deleting or adding at least one amino acid residue.

20. The peptide fragment of claim 1 comprising, for each specific HLA allele, any of the amino acid residues as indicated in the following table:

HLA allele	Position 1	Position 2	Position 3	Position 5	Position 6	Position 7	C-terminal
HLA-A1		T,S	D,E			L	Y
HLA-A2		L, M			V		L,V
HLA-A3		L,V,M	F,Y				K, Y, F
HLA-A11		V,I,F,Y	M,L,F,Y,I				K, R
HLA-A23		I,Y					W,I
HLA-A24		Y		I,V	F		I,L,F
HLA-A25		M,A,T	I				W
HLA-A26	E,D	V,T,I,L,F			I,L,V		Y,F
HLA-A28	E,D	V,A,L					A,R
HLA-A29		E					Y,L
HLA-A30		Y,L,F,V					Y
HLA-A31			L,M,F,Y				R
HLA-A32		I,L					W
HLA-A33		Y,I,L,V					R
HLA-A34		V,L					R
HLA-A66	E,D	T,V					R,K
HLA-A68	E,D	T,V					R,K

HLA-A69		V,T,A					V,L
HLA-A74		T					V,L
HLA-B5		A,P	F,Y				I,L
HLA-B7		P					L,F
HLA-B8			K	K,R			L
HLA-B14		R,K					L,V
HLA-B15 (B62)		Q,L,K,P, H,V,I,M, S,T					F,Y,W
HLA-B17							L,V
HLA-B27		R					Y, K,F,L
HLA-B35		P					I, L, M, Y
HLA-B37		D,E					I,L,M
HLA-B38		H	D,E				F,L
HLA-B39		R,H					L,F
HLA-B40 (B60,61)		E	F,I,V				L,V,A,W, M,T,R
HLA-B42		L,P					Y,L
HLA-B44		E					F,Y,W
HLA-B46		M,I,L,V					Y,F
HLA-B48		Q,K					L
HLA-B51		A,P,G					F,Y,I,V
HLA-B52		Q	F,Y				I,V
HLA-B53		P					W,F,L
HLA-B54		P					
HLA-B55		P					A,V
HLA-B56		P					A,V
HLA-B57		A,T,S					F,W,Y
HLA-B58		A,T,S					F,W,Y
HLA-B67		P					L
HLA-B73		R					P
HLA-Cw1		A,L					L
HLA-Cw2		A,L					F,Y
HLA-Cw3		A,L					L,M
HLA-Cw4		Y,P,F					L,M,F,Y
HLA-Cw6		Y					L,Y,F,Y

HLA-Cw8		Y					L,I,
HLA-Cw16		A,L					L,V

21. The peptide fragment of claim 1 that is capable of eliciting INF-γ -producing cells in a PBL population of a cancer patient at a frequency of at least 10 per 10^4 PBLs.

22. The peptide fragment of claim 1, which is capable of eliciting INF-γ -producing cells in a PBL population of a patient having a cancer disease where a protein belonging to the Bcl-2 protein family is expressed.

23. The peptide fragment of claim 22 where the cancer disease is selected from the group consisting of a haematopoietic malignancy including chronic lymphatic leukemia and chronic myeloid leukemia, melanoma, breast cancer, cervix cancer, ovary cancer, lung cancer, colon cancer, pancreas cancer and prostate cancer.

24. A pharmaceutical composition comprising the protein and/or the peptide fragment of any of claims 1-23.

25. The composition of claim 24 that comprises a peptide fragment according to claim 8 in combination with a peptide fragment according to claim 12.

26. The composition of claim 24, which is a vaccine, which, when it is administered to a cancer patient, is capable of eliciting an immune response against the cancer disease.

27. The composition of claim 26 which is a vaccine, which, when administered to a cancer patient where a Bcl-2 protein family member is expressed, is capable of eliciting an immune response against the cancer disease

28. The composition of claim 27 where during cancer progression the cancer cells have developed a reduced susceptibility to a chemotherapeutically active anti-cancer drug or the radiotherapy.

29. The composition of claim 26 where the cancer disease is selected from the group consisting of a haematopoietic malignancy including chronic lymphatic leukemia and chronic myeloid leukemia, melanoma, breast cancer, cervix cancer, ovary cancer, lung cancer, colon cancer, pancreas cancer and prostate cancer.

30.The composition of claim 26 where the vaccine elicits the production in the vaccinated patient of effector T-cells having a cytotoxic effect against the cancer cells.

31. The composition of claim 24 further comprising an immunogenic protein or peptide fragment selected from a protein or peptide fragment not belonging to or derived from the Bcl-2 protein family.

32. The composition of claim 31 where the protein or peptide fragment not belonging to or derived from the Bcl-2 protein family is a protein involved in regulation of cell apoptosis or a peptide fragment derived therefrom.

33. The composition of claim 31 where the immunogenic protein or peptide fragment selected from a protein or peptide fragment not belonging to or derived from the Bcl-2 protein family is survivin or a peptide fragment hereof.

34. A composition for *ex vivo* or *in situ* diagnosis of the presence in a cancer patient of T cells in PBL or in tumor tissue that are reactive with a Bcl-2 protein family member, the composition comprising a peptide fragment of claim 1.

35. A diagnostic kit for *ex vivo* or *in situ* diagnosis of the presence in a cancer patient of T cells in PBL or in tumour tissue that are reactive with a Bcl-2 protein family member, the kit comprising a peptide fragment according to claim 1.

36. A complex of a peptide fragment of claim 1 and a Class I HLA molecule or a fragment of such molecule.

37. The complex of claim 36 which is monomeric.

38. The complex of claim 36 which is multimeric.

39. A method of detecting in a cancer patient the presence of a Bcl-2 protein family member reactive T-cells, the method comprising contacting a tumour tissue or a blood sample with a complex of claim 36 and detecting binding of the complex to the tissue or the blood cells.

40. A molecule that is capable of binding specifically to a peptide fragment according to claim 1.

41. The molecule of claim 40 which is an antibody or a fragment hereof.

42. A molecule that is capable of blocking the binding of the molecule of claim 40 or 41.

43. A method of treating a cancer disease, the method comprising administering to a patient suffering from the disease an effective amount of the composition of claim 24, the molecule of claim 40 or the molecule of claim 42.

44. The method of claim 43 wherein the disease to be treated is a cancer disease where a Bcl-2 protein family member is expressed.

45. The method of claim 43 wherein the cancer disease is selected from the group consisting of a haematopoietic malignancy including chronic lymphatic leukemia and chronic myeloid leukemia, melanoma, breast cancer, cervix cancer, ovary cancer, lung cancer, colon cancer, pancreas cancer and prostate cancer.

46. The method of to claim 43, which is combined with a further cancer treatment.

47. The method of claim 43 wherein the further treatment is selected from the group consisting of chemotherapy, radiotheraphy, treatment with immunostimulating substances, gene therapy, treatment with antibodies and treatment using dendritic cells

48. Use of the protein or peptide fragment of any of claims 1-23 in the manufacturing of a medicament for the treatment of a cancer disease.

49. The use of claim 48 wherein the disease to be treated is a cancer disease where a Bcl-2 protein family member is expressed.

50. The use of claim 49 wherein the cancer disease is selected from the group consisting of a haematopoietic malignancy including chronic lymphatic leukemia and chronic myeloid leukemia, melanoma, breast cancer, cervix cancer, ovary cancer, lung cancer, colon cancer, pancreas cancer and prostate cancer.

51. The use of claim 48, which is combined with a further cancer treatment.

52. The use of claim 51 wherein the further treatment is selected from the group consisting of chemotherapy, radiotheraphy, treatment with immunostimulating substances, gene therapy, treatment with antibodies and treatment using dendritic cells.

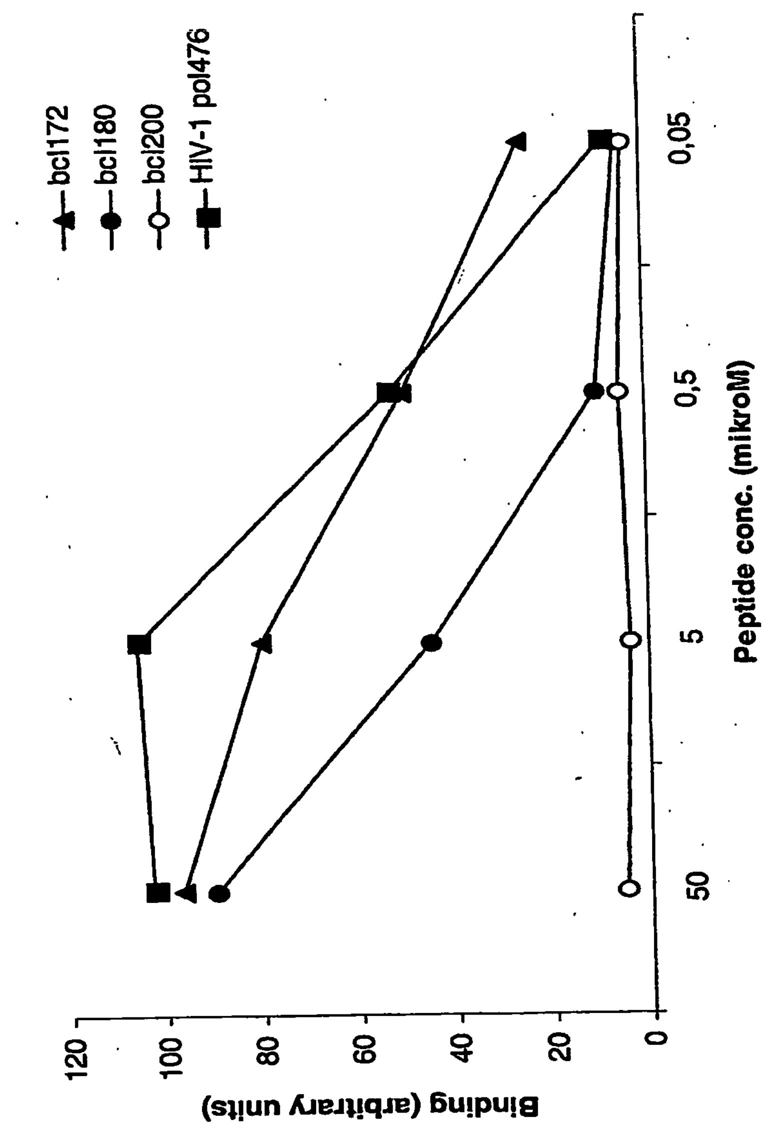
bcl172
bcl180
bcl200
HIV-1 pol476
Binding (arbitrary units)
120
100
80
60
40
20
0
50
5
0,5
0,05
Peptide conc. (mikroM)

Fig. 1

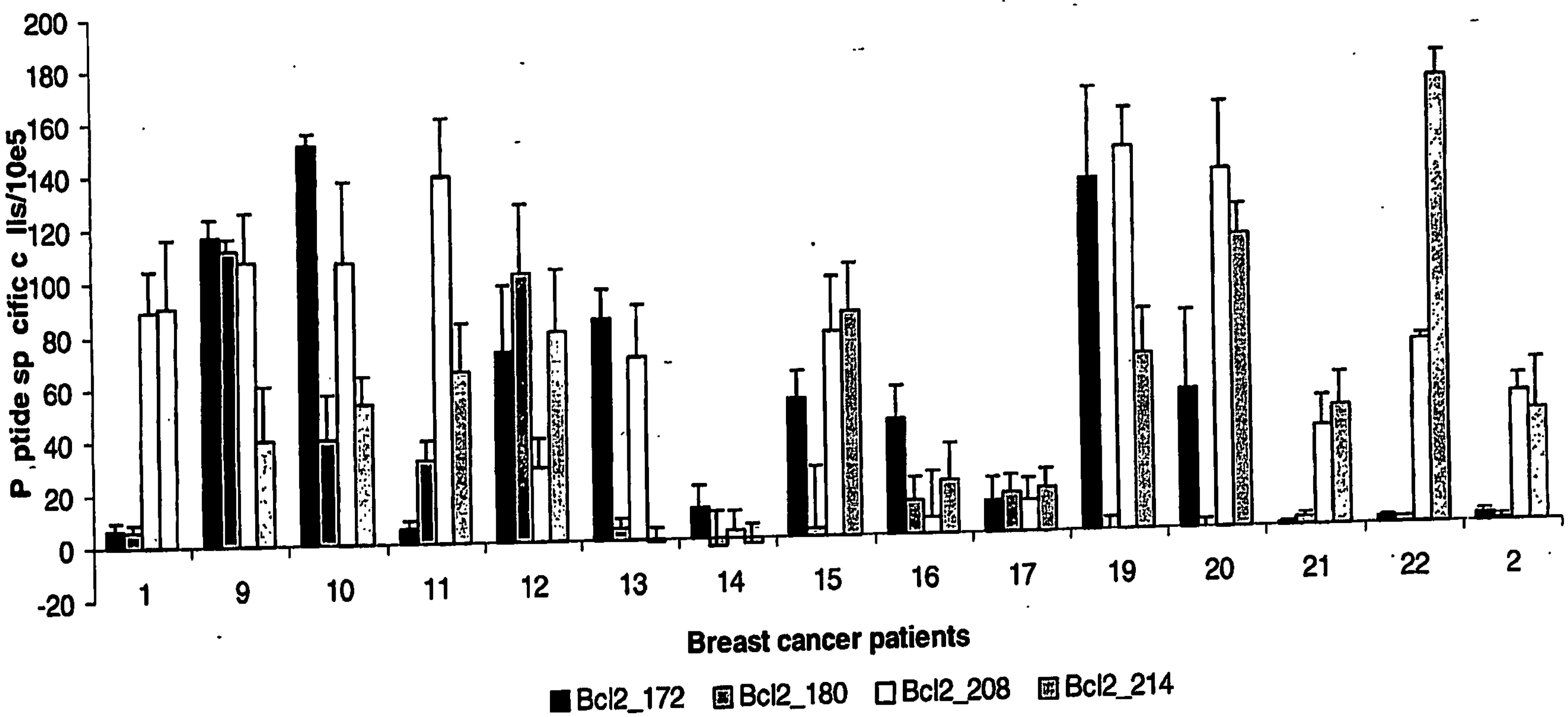
P ptide sp cific c lls/10e5
200
180
160
140
120
100
80
60
40
20
0
-20
1
9
10
11
12
13
14
15
16
17
19
20
21
22
2
Breast cancer patients
Bcl2_172
Bcl2_180
Bcl2_208
Bcl2_214

Fig. 2

Document made available under the Patent Cooperation Treaty (PCT)

International application number: PCT/DK04/000799

International filing date: 18 November 2004 (18.11.2004)

Document type: Certified copy of priority document

Document details: Country/Office: US
Number: 60/523,119
Filing date: 19 November 2003 (19.11.2003)

Date of receipt at the International Bureau: 25 January 2005 (25.01.2005)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)